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# Platelet-derived growth factor induces apoptosis in vascular smooth muscle cells: roles of the Bcl-2 family

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**Abstract**

Apoptosis (programmed cell death) is observed in vascular smooth muscle cells (VSMC) in atherosclerotic lesions and stenotic lesions after injury, and modulates the cellularity of these lesions. It is recognized that cell growth and apoptosis are two linked processes. Platelet-derived growth factor (PDGF) induces VSMC proliferation and migration in vitro. We studied the effect of PDGF on apoptosis in VSMC. Cultured rat VSMC were treated with PDGF-AA or PDGF-BB. PDGF-BB induced cell death in cultured VSMC in a time- and dose-dependent manner, but PDGF-AA did not. Gel electrophoresis of genomic DNA and in situ DNA labeling confirmed that the cell death induced by PDGF-BB is apoptosis. PDGF-BB treatment reduced bcl-2 mRNA and bcl-xl mRNA expression, in contrast, induced bcl-xs mRNA expression, linked with the induction of apoptosis in cultured VSMC. 

*Keywords:* Apoptosis; Platelet-derived growth factor; Vascular smooth muscle cell; Bcl-2 family

## 1. Introduction

Apoptosis plays an important role in normal development and many pathological processes. Induction of apoptosis is shown in a variety of diseases including cancer, neurological disorders, and cardiovascular diseases [1–4]. Cell death is recognized in atherosclerotic lesions and stenotic lesions after balloon injury [5–7] in which excessive accumulation of VSMC occurs. Apoptosis has also been observed in the heart of spontaneously hypertensive rats [8] and in the hypertrophied heart induced by pressure overload [9]. These observations indicate that cell growth and apoptosis are two tightly linked processes in cardiovascular diseases.

Platelet-derived growth factor (PDGF) has been shown to be involved in the accumulation of VSMC as chemoattractant after balloon injury of the normal rat carotid artery in vivo [10]. PDGF-BB induces proliferation and migration of VSMC in vitro [11,12]. PDGF induces other important cellular responses, including survival [13] and transformation [14]. In this study, we showed that PDGF-BB induced apoptosis in cultured VSMC.

Apoptosis is regulated by a genetic program [15]. The Bcl-2 family is an important regulator of cell death acting as a suppressor or an inducer [16]. In transfection experiments, constitutive expression of high levels of Bcl-2 is able to protect many cell types

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from apoptosis induced by exposure to a wide variety of adverse conditions and stimuli [16–19]. Several additional proteins homologous to Bcl-2 have been identified. One of these proteins, Bax, has the opposite effect to Bcl-2, and overexpression of Bax accelerates apoptotic death [20]. Bcl-x is another member of the Bcl-2 family [21]. From a single *bcl-x* gene, alternative splicing produces two mRNA species with different sizes (707 base pairs (bp) and 518 bp in the coding region). When the longer mRNA, which translates Bcl-xl, is overexpressed in cells, apoptosis is suppressed in a manner analogous to the action of Bcl-2. In contrast, when the short mRNA, which translates Bcl-xs, is overexpressed in cells, apoptosis is induced in a manner analogous to the action of *Bax*.

In recent studies, overexpression of c-Myc [22,23], interleukin-1β [24], and antioxidant [25] induced apoptosis in VSMC. However, the precise mechanisms of apoptosis in VSMC have not been elucidated. In this study, we report that the Bcl-2 family regulates PDGF-BB-induced apoptosis in cultured VSMC.

## 2. Materials and methods

### 2.1. Isolation and culture of vascular smooth muscle cells

Rat VSMC (passage 3–10) were isolated from thoracic aortas of 10-week-old Sprague-Dawley rats (Charles River Japan, Kanagawa, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) and maintained at 37°C with atmospheric air and 5% $CO_2$. After reaching confluence, the cells were transferred to DMEM containing 0.1% FCS and cultured for 24 h, and then these cells were treated in freshly prepared DMEM-0.1% FCS without (as a control) or with PDGF (Upstate Biotechnology, Lake Placid, NY, USA) for an additional 96 h. PDGF were added to the culture medium at a concentration of 100 ng/ml for PDGF-AA and PDGF-BB. Cell morphology was examined by phase contrast microscopy, and microphotographs were taken during incubation with PDGF-AA or PDGF-BB.

### 2.2. Flow cytometry and cell death

Cell death was determined by cell cycle analysis using a fluorescence-activated cell sorter flow cytometry (FACS). VSMC were cultured in 12-well plates in DMEM containing 10% FCS until confluent density and then cultured in DMEM with 0.1% FCS for 24 h. The cells were treated with PDGF-BB at the indicated concentrations. After treatment with PDGF-BB for the indicated times, the cells were trypsinized and stained with propidium iodide using a Cycle Test Plus DNA Reagent Kit (Becton Dickinson, San Jose, CA, USA). The stained cells were then subjected to flow cytometry on the FACS (FACSCalibur cytofluorometer, Becton Dickinson) and analyzed with CELL Quest software (Becton Dickinson). Cell cycle analysis indicates that the cells in $G_1$, S and $G_2$/M phases are alive and the cells in pre-$G_1$ phase (apoptotic phase) are dead. The percentage of cell death was calculated by the cell number in pre-$G_1$ phase/the total cell number $\times 100$.

### 2.3. Cell proliferation assay

Cell proliferation assay was performed using cell proliferation reagent WST-1 (Boehringer Mannheim, Mannheim, Germany). The cleavage of tetrazolium salts, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases resulted in formazan in viable cells, which was used for the quantification of cell proliferation and cell viability by a colorimetric assay. VSMC were seeded onto 96-well tissue culture plates at a concentration of $2 \times 10^3$/well in 100 μl of DMEM containing 10% FCS and cultured for 48 h (20–30% confluence). The cells were transferred to DMEM with 0.1% FCS and cultured for 24 h, and then treated with 100 ng/ml of PDGF-BB for the indicated times. After treatment, 10 μl of WST-1 were added to each well and the plate was incubated for 1 h. Absorbance was measured at 450 nm with reference wavelength at 690 nm by ELISA reader (Multiskan Bichromatic, Labsystems, Helsinki, Finland).

### 2.4. DNA fragmentation analysis

Genomic DNA was isolated from VSMC after treatment with PDGF-AA or PDGF-BB for 96 h. Cells were lysed in 500 μl DNA extraction solution containing 20 mmol/l Tris-HCl, pH 7.4, 0.1 mol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium dodecyl sulfate (SDS). Cell lysates were incubated with 100 μg/ml proteinase K (Ambion, Austin, TX, USA) for 16 h at 37°C. After incubation, 500 μl phenol was added to the cell lysates, and mixed gently for 6 h. The mixture was centrifuged at 15000 rpm for 20 min, and the upper aqueous phase was incubated with 5 μg/ml RNase for 1 h at 37°C. After phenol/chloroform (1:1, v/v) treatment, DNA was precipitated and dissolved in 10 mmol/l Tris-HCl and 1 mmol/l EDTA. Finally, DNA was electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

### 2.5. In situ DNA labeling analysis

To detect DNA fragmentation in situ, nick end labeling was performed using an ApoTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). VSMC on a chamber slide were fixed with 0.25% glutaraldehyde and treated with 0.3% $H_2O_2$ for 30 min for inactivation of endogenous peroxidase, and then incubated with digoxygenin-conjugated dUTP and deoxyribonucleotide transferase for 1 h at 37°C. Labeled DNA fragments were incubated with antidigoxygenin monoclonal antibody linked with peroxidase for 30 min at room temperature. The chromogen 3,3′-diaminobenzidine (DAB) (Wako Pure Chemical Industries, Osaka, Japan) was used as substrate against peroxidase.

### 2.6. Isolation of cDNA for bcl-2, bax, and bcl-x

Total RNA was isolated from VSMC according to the manufacturer's instructions with Isogen (Nippon Gene, Tokyo, Japan), and reverse-transcribed into first strand cDNA with oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (RT) using a first strand cDNA synthesis kit (Clontech, Palo Alto, CA, USA). Specific sense and anti-sense primers for rat *bcl-2* (sense: 5′-ATGGCGCAAGCCGGGAGAAC-3′; anti-sense: 5′-TCACTTGTGGCCCAGGTATG-3′), *bax* (sense: 5′-GACACCTGAGCTGACCTTGG-3′; anti-sense: 5′-GAGGAAGTCCAGTGTCCAGC-3′), and *bcl-x* (sense: 5′-ATGTCTCAGAGCAACCGGGA-3′; anti-sense: 5′-TCACTTCCGACTGAAGAGTG-3′) were synthesized, and the first strand cDNA was subjected to PCR amplification. PCR conditions were denaturation at 94°C for 45 s, annealing at 55°C for 45 s and elongation at 72°C for 60 s, for 30 cycles. Amplified products were electrophoresed on 0.8% agarose gel and purified. Purified DNA fragments were subcloned into Bluescript KS(+) II plasmid vector (Stratagene, La Jolla, CA, USA). Sequence analysis was carried out on both strands.

### 2.7. Northern blot analysis

Total RNA isolated from VSMC was electrophoresed on 1.0% agarose gel, and transferred to nylon membranes (Highbond-$N^+$, Amersham, Bucks, UK). Hybridization was performed using an ExpressHyb solution (Clontech) according to the manufacturer's instructions. $^{32}$P-labeled cDNA probes were prepared by the random primer method using a Prime-It II random primer labeling kit (Stratagene). After washing, autoradiography was performed with X-ray film (Hyperfilm-MP, Amersham) with an intensifying screen at −80°C.

### 2.8. RT-PCR analysis of bcl-xl and bcl-xs mRNA expression levels

To confirm the result by Northern blotting, the RT-PCR method was used to measure the mRNA expression level of *bcl-xl* or *bcl-xs*. Total RNA (1 μg each) from VSMC was reverse-transcribed into first strand cDNA using oligo(dT) primers. The synthesized first strand cDNA was subjected to 30 cycle PCR amplification using the specific primers encoding rat *bcl-x* or rat GAPDH (sense: 5′-TGGAGTCTACTGGCGTCTTC-3′; anti-sense: 5′-CAAAGGTGGAGGAATGGGAG-3′). PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. Amplified products (*bcl-xl*: 707 bp, *bcl-xs*: 518 bp) were identified by sequence analysis.

## 3. Results

### 3.1. Cell death induced by PDGF-BB in 100% confluent VSMC

After incubation with PDGF-AA or PDGF-BB for 96 h, morphological changes were examined by phase contrast microscopy. Morphological changes were not observed in cells treated with PDGF-AA or in control cells (DMEM-0.1% FCS) (Fig. 1A,B). In contrast, in PDGF-BB-treated cells, some cells floated, shrank and became round. Cell membrane was blebbed and cytoplasm condensed (Fig. 1C). Flow cytometric analysis demonstrated that cell death occurred in PDGF-BB-treated cells in a time- and dose-dependent manner (Fig. 2A,B).

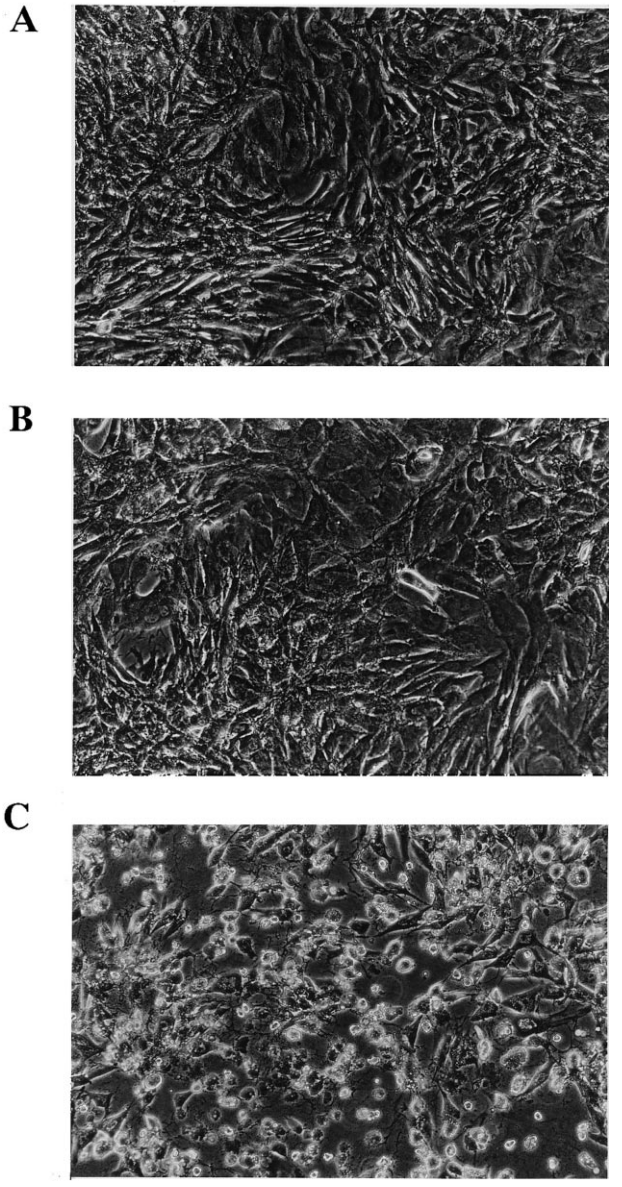


Fig. 1. Apoptotic features of PDGF-BB-induced cell death. Quiescent cultured VSMC were incubated with PDGF-AA or PDGF-BB. After 96 h, cells were observed by phase-contrast microscopy (original magnification: ×150). (A) Medium containing 0.1% FCS alone as a control, (B) 100 ng/ml PDGF-AA, (C) 100 ng/ml PDGF-BB.

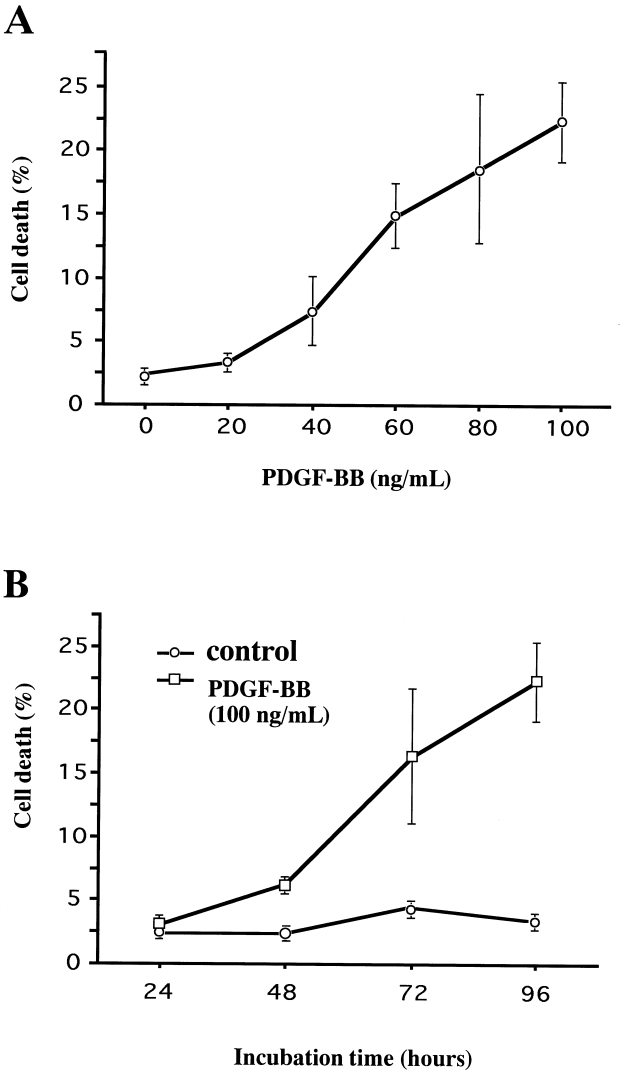


Fig. 2. Time- and dose-dependent manner of induction of apoptosis by PDGF-BB. (A) VSMC were treated with 100 ng/ml of PDGF-BB for the indicated times. (B) VSMC were treated with PDGF-BB at the indicated concentrations for 96 h. After treatment, cells were harvested for FACS analysis. The results represent the mean ± S.D. of three experiments.

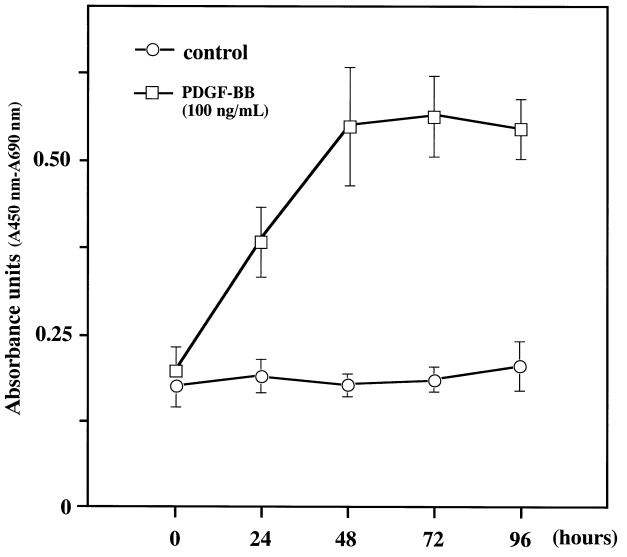


Fig. 3. Cell proliferation by PDGF-BB in non-confluent VSMC. VSMC at 20–30% confluence were treated with 100 ng/ml of PDGF-BB for the indicated times. After treatment, cell proliferation reagent, WST-1, was added to the medium. Absorbance was measured at 450 nm with reference wavelength at 690 nm by ELISA reader. The results represent the mean ± S.D. of six experiments.

### 3.2. Cell proliferation induced by PDGF-BB in non-confluent VSMC

Although PDGF-BB induced cell death in VSMC at 100% confluence, it induced cell proliferation in VSMC at 20–30% confluence for 48 h as shown in Fig. 3. This showed the usual proliferation effect of PDGF-BB on VSMC.

### 3.3. DNA fragmentation in VSMC undergoing apoptosis

Apoptosis was confirmed by the characteristic laddering pattern of nuclear chromatin fragmentation on the electrophoresis of genomic DNA. Genomic DNA from the control and PDGF-AA-treated cells showed only high molecular DNA. In contrast, internucleosomal DNA (about 180 bp) appeared in VSMC treated with PDGF-BB, indicating that apoptosis occurred in PDGF-BB-treated cells (Fig. 4A). We also evaluated DNA fragmentation in situ, using a nick end labeling method. Cells treated with PDGF-BB showed positive nuclear staining with DAB, mainly in rounded cells (Fig. 4B). Stained nuclei were condensed and fragmented.

### 3.4. Gene expression of the Bcl-2 family

To explore the possibility that the Bcl-2 family plays a role in apoptosis in VSMC induced by PDGF-BB, we quantified mRNA expression levels of *bcl-2*, *bax*, *bcl-xl*, and *bcl-xs* by Northern blot analysis. *bcl-2* mRNA was clearly detected in control

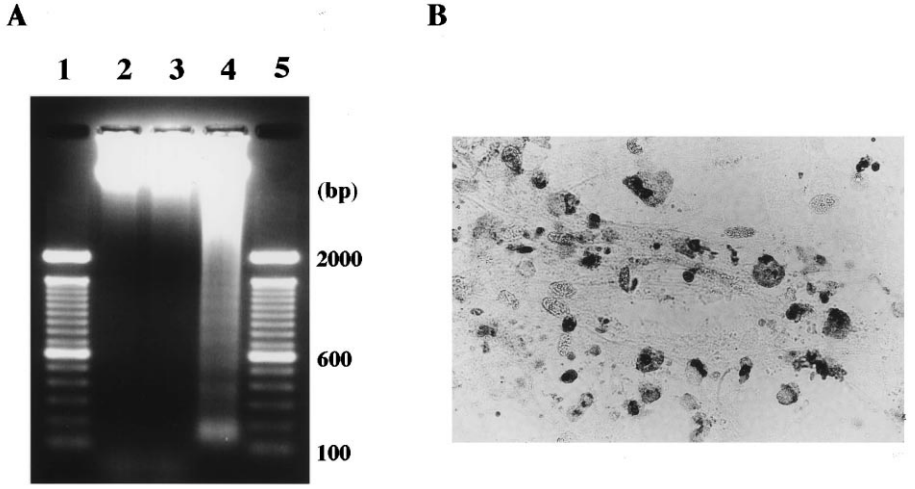


Fig. 4. (A) Gel electrophoresis of internucleosomal DNA fragmentation. Genomic DNA was extracted from VSMC treated with PDGF-AA or PDGF-BB for 96 h. DNA (5 μg/lane) was electrophoresed on 1.5% agarose gel. Lanes: 2, medium containing 0.1% FCS alone as a control; 3, 100 ng/ml PDGF-AA; 4, 100 ng/ml PDGF-BB; 1 and 5, DNA size markers. (B) Identification of apoptotic cells by in situ nick DNA end labeling. Quiescent VSMC were incubated with 100 ng/ml PDGF-BB for 72 h in a chamber slide (Original magnification: ×400).

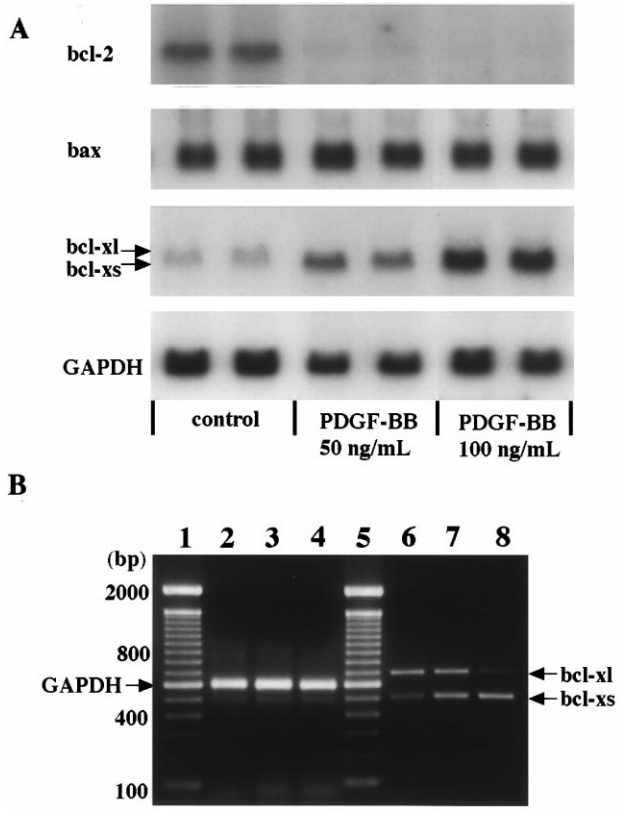


Fig. 5. Effects of PDGF-BB-induced apoptosis on *bcl-2*, *bax* and *bcl-x* mRNA expression in VSMC. (A) Northern blot analysis shows bands of *bcl-2*, *bax*, *bcl-x* and GAPDH mRNA from VSMC treated with medium containing 0.1% FCS alone as a control or 50 or 100 ng/ml PDGF-BB. Results are representative of three experiments. (B) Effects of PDGF-BB-induced apoptosis on *bcl-xl* and *bcl-xs* mRNA by the RT-PCR method. Agarose gel electrophoresis shows RT-PCR products using *bcl-x* or GAPDH specific primers from VSMC treated with medium containing 0.1% FCS alone as control (lanes 2 and 6), 50 ng/ml PDGF-BB (lanes 3 and 7) or 100 ng/ml PDGF-BB (lanes 4 and 8) for 96 h. Lanes 1 and 5 show DNA size markers.

VSMC, however, PDGF-BB at both 50 and 100 ng/ml reduced *bcl-2* mRNA expression to almost undetectable levels (Fig. 5A). In contrast, there were no apparent differences in *bax* mRNA levels among the three groups (Fig. 5A). In the analysis of *bcl-x* mRNA levels, control cells expressed *bcl-xl* mRNA but not *bcl-xs* mRNA. However, following treatment with PDGF-BB, the *bcl-xl* mRNA level was drastically reduced, in contrast with the increase of the *bcl-xs* mRNA level (Fig. 5A). Two forms of *bcl-x* mRNA, *bcl-xl* and *bcl-xs* mRNA, were able to be distinguished by Northern blot analysis. However, since the two forms of *bcl-x* mRNA differ in size by only 189 bp, *bcl-xl* and *bcl-xs* mRNA levels were also determined using an RT-PCR method. In control cells, both *bcl-xl* and *bcl-xs* mRNA could be detected by the RT-PCR method, but *bcl-xl* mRNA was expressed at a higher level than *bcl-xs* mRNA. The *bcl-xl* mRNA level was reduced and the *bcl-xs* mRNA level increased by induction of apoptosis by PDGF-BB (Fig. 5B).

## 4. Discussion

It is widely accepted that apoptosis requires exogenous signals and endogenous genes which also lead to differentiation and proliferation [15,16]. Recent studies have demonstrated that apoptosis is induced in atherosclerotic lesions as well as restenotic lesions after balloon injury [5–7]. In these lesions, migration and proliferation of VSMC are promoted by diverse growth factors including PDGF [10,11]. In the present study, we showed that PDGF induced apoptosis in cultured VSMC, and analyzed the mechanism of PDGF-induced apoptosis.

Kim et al. [26] used PDGF to induce apoptosis in growth arrested murine fibroblasts. In their study, reverse growth arrest was induced in the $G_0$ phase by serum deprivation in murine fibroblasts. The addition of PDGF to fibroblasts can stimulate expression of c-*myc*, an immediate early response gene [27]. Activation of c-Myc expression leads to an accumulation of p53 protein by stabilization in quiescent fibroblasts [28]. The accumulation of c-Myc and/or p53 can induce apoptosis in fibroblasts [28–30]. From these observations, they speculated that c-Myc and p53 are key factors in initiating apoptosis in murine fibroblasts. In our experiments, since confluent VSMC were cultured in 0.1% FCS for 24 h and then cell death was induced by PDGF-BB, c-Myc and/or p53 may be involved in apoptosis in VSMC as well as in fibroblasts [26]. The difference between our study and their study is the cell confluence on PDGF treatment. They treated the fibroblasts at 10% confluence with PDGF to induce apoptosis. We also treated VSMC with PDGF-BB before reaching confluence (20–30% confluence). PDGF-BB induced cell proliferation for 48 h and did not induce apoptosis for 96 h. This result strongly indicates that

cell density at 100% confluence is one of the essential factors for induction of apoptosis in VSMC. Cell-cell contact (contact inhibition) leads to cell cycle arrest [31]. Contact inhibition may be a critical factor in PDGF-BB induced apoptosis in VSMC.

We demonstrated that apoptosis in VSMC was able to be induced by PDGF-BB but not PDGF-AA. We could detect the expression of PDGF-β receptor, but not PDGF-α receptor, by Northern blot analysis in VSMC derived from Sprague-Dawley rats (data not shown). This finding is consistent with the report by Kitami et al. [32]. They demonstrated that VSMC derived from normotensive Wistar Kyoto rats expressed PDGF-β receptor but not PDGF-α receptor. PDGF-AA specifically binds to the PDGF-α receptor but does not bind to the PDGF-β receptor, whereas PDGF-BB binds to both PDGF-α and PDGF-β receptors [33,34]. These observations indicate that PDGF-BB induces an apoptotic signal in VSMC specifically via PDGF-β receptor. The failure of PDGF-AA to induce apoptosis in VSMC may be due to the absence of the appropriate receptor. Inui et al. [35] reported differences in signal transduction between PDGF-α and PDGF-β receptors in VSMC. PDGF-BB strongly induced both protein and DNA synthesis in VSMC, whereas PDGF-AA induced only protein synthesis even if they used the VSMC with highly expressed PDGF-α receptor. In VSMC, PDGF-BB acts as a potent mitogen but PDGF-AA does not in vitro [36]. The induction of apoptosis in VSMC by PDGF-BB may depend on its mitogenic activity.

The *bcl-2* protooncogene was originally identified as a transcriptional factor with the t(14:18) chromosomal translocation found in follicular lymphomas [37]. Bcl-2 promotes survival under apoptotic stimulus in various cells including hematopoietic cells [17], neurons [19], endothelial cells [38] and cultured VSMC [39]. Nishio et al. [39] used oxysterols, 7-ketocholesterol or 25-hydroxycholesterol to induce apoptosis in rabbit VSMC. Exposure to oxysterols decreased the expression of Bcl-2 in VSMC undergoing apoptosis. In our study, the *bcl-2* mRNA level was decreased by induction of apoptosis in VSMC. This observation is in agreement with the report by Nishio et al. [39] as well as with previous studies demonstrating prevention of apoptosis in *bcl-2*-transfected cells [17–19].

Several novel proteins homologous to Bcl-2 have been isolated. Bax has been identified as a Bcl-2-associated protein, and it has been shown that its overexpression accelerates apoptotic death induced by cytokine deprivation in an interleukin-3-dependent cell line [20]. The ratio of the expression level of Bcl-2 to Bax determines survival or death after an apoptotic stimulus. When Bcl-2 is in excess, cells are protected from death. On the other hand, when Bax is in excess, cells are susceptible to apoptosis [20]. In our study, the induction of apoptosis by PDGF-BB did not affect the *bax* mRNA level but reduced the *bcl-2* mRNA level, resulting in a shift to a relatively higher *bax* mRNA level than *bcl-2* mRNA level.

Bcl-x has been identified as a Bcl-2 homolog. By alternative splicing, two mRNAs that differ in size by 189 nucleotides are transcribed from a single gene *bcl-x* [21]. Sedlak et al. [40] have reported that Bax is dimerized with Bcl-xl and speculated that a change in the ratio of Bcl-2 and Bcl-xl to Bax and Bcl-xs plays a predominant role in determining either survival (higher Bcl-2 and Bcl-xl expression) or death (higher Bax and Bcl-xs expression). We further examined changes in *bcl-x* mRNA levels after the apoptotic stimulus of PDGF-BB. In control cells, a higher level of *bcl-xl* mRNA than of *bcl-xs* mRNA was expressed. However, following treatment with PDGF-BB, *bcl-xl* mRNA was decreased and *bcl-xs* mRNA was increased. Switching from *bcl-xl* to *bcl-xs* mRNA resulted in higher levels of *bax* and *bcl-xs* mRNA than of *bcl-2* and *bcl-xl* mRNA. The increased relative expression ratio of *bax* and *bcl-xs* mRNA may be a critical factor in induction of apoptosis in VSMC.

Perlman et al. [41] reported that medial VSMC in balloon-injured rat carotid arteries were induced apoptosis and the induction of apoptosis coincided with a marked downregulation of Bcl-x (= Bcl-xl; they said that *bcl-x* confers protection from apoptosis in their study) expression. They also showed no change in Bax expression in apoptotic VSMC. It is not clear what regulates Bcl-x expression in VSMC in balloon-injured arteries. However, their results are in agreement with our results that the Bcl-2 family regulates apoptosis in VSMC. PDGF may regulate Bcl-2 family expression and apoptosis in VSMC in balloon-injured arteries.

In conclusion, PDGF-BB induced apoptosis in cul-

tured VSMC. The induction of apoptosis in VSMC may be linked with a shift of the ratio of *bcl-2* to *bax* mRNA as well as *bcl-xl* to *bcl-xs* mRNA. These results provide a great insight into the mechanism of apoptosis in VSMC.

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